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Combining cadherin expression with molecular markers discriminates invasiveness in GH and PRL pituitary adenomas

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Declaration of interest

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Abstract

Although GH- and PRL-secreting pituitary adenomas are considered benign, in many patients, tumor growth and/or invasion constitute a particular challenge. In other tumors, progression relies in part on dysfunction of intercellular adhesion mediated by the large family of cadherins. In this study, we have explored the contribution of cadherins in GH and PRL adenoma pathogenesis, and evaluated whether this class of adherence molecules was related to tumor invasiveness. We have first established, by qPCR and immunohistochemistry, the expression profile of classical cadherins in normal human pituitary gland. We show that the cadherin repertoire is restricted and cell-type specific. Somatotrophs and lactotrophs express mainly E-cadherin and cadherin 18, while N-cadherin is present in the other endocrine cell types. This repertoire undergoes major differential modification in GH and PRL tumors: E-cadherin is significantly reduced in invasive GH adenomas, and this loss is associated with a cytoplasmic relocalization of cadherin 18 and catenins. In invasive prolactinomas, E-cadherin distribution is altered and is accompanied by a mislocalization of cadherin 18, beta-catenin and p120 catenin. Strikingly, de novo expression of N-cadherin is present in a subset of adenomas and cells exhibit a mesenchymal phenotype exclusively in invasive tumors. Binary tree analysis, performed by combining the cadherin repertoire with the expression of a subset of known molecular markers, shows that cadherin/catenin complexes play a significant role in discrimination of tumor invasion.

Introduction

Pituitary tumors represent up to 10-15% of intracranial neoplasms with a prevalence of 0.1% in the overall population (1). These common tumors display various clinical behaviors and most are sporadic, however familial isolated pituitary adenomas have been described (1, 2).

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Although usually benign, they cause significant morbidity due to mass effects, local expansion and/or hormonal deficiency or excess. Among the various pituitary tumor subtypes, prolactinomas are the most common, accounting for up to 45% of pituitary adenomas presenting at clinic (3). Prolactinomas are easily controlled in the majority of cases by pharmacological treatment with dopamine agonists. However, invasive prolactinomas can be resistant to dopamine agonist treatment and have lower cure and higher recurrence rates compared with non-invasive prolactinomas (4). GH-secreting adenomas represent 10-15% of pituitary adenomas (5) and cause acromegaly and gigantism. Various treatment options are available for acromegaly, including for medical therapy, somatostatin analogs, dopamine agonists, and GH receptor antagonists. Several mechanisms and predictors for somatostatin resistance have been reported to date (6) and involve, for example, alterations in somatostatin receptor subtype expression patterns (7) and a decrease in E-cadherin expression (8). Most pituitary tumors in acromegalic patients are benign, nevertheless, many tumors can behave more aggressively and require multiple treatments (9).

The prediction of invasive behavior in pituitary adenomas is complex and challenging, and many studies are dedicated to the identification of potential biomarkers (10-13). In a recent retrospective multicentre case–control study, invasion has been shown to be a major prognostic factor in predicting recurrence/progression following treatment of pituitary tumors by surgery (14). To date, no single marker has been identified to independently predict tumor behavior, although some markers appear to be specific for different adenoma subtypes. Indeed, large scale studies have shown that a combination of markers is more useful to predict tumor behavior, especially aggressiveness (15). However, the identification of other biomarkers is still necessary and would help optimization of therapeutic strategies. The tumor microenvironment, in particular intercellular adhesion, plays a key role during tumor progression and invasion, and cadherin/catenin complex dysfunction is a major contributor to

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cancer progression. Abnormal or reduced expression of these complexes is observed in various cancers (16). For instance, the loss of E-cadherin is a hallmark of many epithelial cancers and has been associated with invasiveness, metastasis, and poor prognosis (16, 17). Loss of E-cadherin is often associated with *de novo* expression of mesenchymal cadherins. This process has multiple consequences for the phenotype and behavior of cells, and constitutes one major aspect of the cancer-related Epithelial Mesenchymal Transition (EMT) process (18). Epithelial E-cadherin expression has been investigated in several pituitary adenoma subtypes and its alteration could contribute to invasiveness of pituitary adenomas (8, 19, 20). In addition, molecular studies have suggested that EMT may play an important role in GH adenoma progression (21-24).

The large superfamily of cadherin proteins includes classical cadherins, protocadherins and cadherin-related molecules. Their specific roles in initiation and progression of many tumors is starting to be elucidated, and has revealed that they can influence tumor behavior by multiple complex mechanisms (16, 25). However, their expression and function in the normal as well as impaired human pituitary gland still needs to be established. We have previously demonstrated that the murine pituitary gland is a highly organized structure (26), and that cadherins are involved in this specialized organization (27). We have shown that a restricted cadherin repertoire is present in the anterior pituitary, and that each cell type expresses a specific subset of cadherins. This repertoire is plastic during pituitary tissue remodeling, especially during development and sexual maturation. These findings and the recent report that abnormal hormone secretion in hyperfunctioning adenomas could be due to spatial and functional disorganization of the pituitary gland (28) led us to hypothesize that alterations in the repertoire of cadherins may contribute to pathology. Here, we first established the pattern of cadherin expression in normal human pituitary gland by screening more than 20 members of the cadherin family. We then examined whether this repertoire was

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modulated in tumors, focusing on 2 major subtypes of pituitary adenomas, GH-secreting adenomas and prolactinomas. A decrease or loss of E-cadherin expression was correlated with invasiveness in GH tumors and was related to a cytoplasmic redistribution of cadherin/catenin complexes. We show that *de novo* expression of N-cadherin takes place in a subset of adenomas, and cells adopt a mesenchymal phenotype unique to invasive tumors. Correlation of cadherin expression with known biomarkers of invasion and binary tree analysis indicates that the cadherin repertoire can differentiate tumor invasive behavior.

Materials and methods

Patients and samples

cDNA from normal pituitary glands (n = 3) was obtained from La Timone Hospital (Marseille, France) and tissue sections from normal pituitary glands (n = 3) were obtained from Legal Medecine Department at Lapeyronie Hospital (Montpellier, France) and Pathological Anatomy and Cytology Departement at Gui De Chauliac Hospital (Montpellier). GH- and PRL-secreting adenoma fragments were obtained from 52 patients who had undergone transsphenoidal surgery at two University hospitals (La Timone Hospital - Marseille, and Pellegrin-Tripode Hospital - Bordeaux, France). Samples from 20 male and 32 female patients (mean age: 39 ± 16 years, range 3 - 68 years) were collected from Pathology Departments by the Tumor Banks of both hospitals after patient information by the physician and registration of consent according to the guidelines of the French Bioethical law for non-interventional research studies. Then, samples were anonymised, distributed for the present research and processed for RNA extraction or tissue sections. The diagnoses of clinical acromegaly or hyperprolactinemia were made according to clinical and hormonal evaluation with additional information provided by histological assessment and immunohistochemical

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staining for all anterior pituitary hormones on tumoral fragments. Tumors were evaluated on the basis of preoperative magnetic resonance imaging scans and defined as invasive on the basis of the cavernous sinus invasion. Based on this parameter, 14 patients had invasive GH-secreting adenoma and 13 patients had non-invasive GH-secreting adenoma, 8 patients had invasive prolactinoma and 17 patients had non-invasive prolactinoma. Patient clinical characteristics are shown in Table S1.

Real-time RT-PCR

Total RNA was extracted and then reverse-transcribed as previously described (29). Specific primers for qRT-PCR were designed using the Primer Express 3.0 software, the sequences are shown in Table S2. Cadherin/catenin expression was analysed on all samples (n=52), and the expression for the other genes was performed on 50/52 samples since the quantity of cDNA was too low for two invasive GH tumors. For gene expression quantification, primers for each gene of interest (5 µl, 300 or 900 nM final) were first evaporated in 384-well plates using a Vacufuge® vacuum concentrator (Eppendorf), then resuspended in 2.5 µl LightCycler*480 SYBR Green 1 Master mix 2X (Roche) and 5 ng of sample RNA in a total reaction volume of 5 µl. PCR reactions were performed by using the LightCycler 480 system with the following conditions: an initial denaturation step for 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 30 sec. Specificity for each PCR reaction was assessed by melting curve analysis of amplicons. For each sample, values were determined from triplicate measurements. Data were analyzed using the threshold cycle (Ct) relative quantification method (LightCycler 480 SW 1.5 software). Expression of gene of interest was normalized to the expression levels of three housekeeping genes *RPLP0*, *RPL31*, and *GAPDH*, according to the formula: 2-[Ct(gene)-arithmetic mean (C*t*(*RPLP0*), Ct(*RPL31*),

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Ct(*GAPDH*))]x10000. Estimation of the stability of references genes was established using the geNorm method (30).

Antibodies, Immunohistochemistry and Confocal microscopy

The primary antibodies and their dilutions used in this study were as follows: mouse monoclonal against E-cadherin (1:150; clone 36B5, ThermoScientific); mouse monoclonal against N-cadherin (1:250; clone 3B9, Zymed Laboratories Inc); rabbit polyclonal against CDH18 (1:25; HPA014365, Sigma), mouse monoclonal against beta-catenin (1:200; clone14, Transduction Laboratories), rabbit polyclonal against beta-catenin (1:50, H102, Santa Cruz Biotechnology), mouse monoclonal against p120 catenin (1:500; pp120 clone98, Transduction Laboratories). Guinea pig polyclonal antibodies against each pituitary hormone (1:5000) were obtained from the National Hormone and Peptide Program, NIDDK and Dr AF Parlow, (Torrance, CA, USA). For cadherins or catenins immunostaining, tyramide signal amplification (TSA Plus Cyanine 3; PerkinElmer) was applied according to the manufacturer's directions. After deparaffinizing and rehydrating, heat-induced epitope retrieval was performed in sodium citrate buffer (0.01 M, pH 6.0). Slides were incubated in 1% hydrogen peroxide to quench endogenous peroxidases and washed in phosphate-buffered saline (PBS). Sections were incubated overnight at 4°C with primary antibodies in PBS containing 0.1% Triton X-100 and 2% bovine serum albumin and 2% normal donkey serum (Sigma). After rinsing in PBS, slides were incubated for 2h with appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). After washing, tissue sections were incubated for 15 min with cyanine 3-conjugated tyramide in the diluent supplied in the kit, carefully washed and mounted in Mowiol (Calbiochem). For double immunofluorescence, cadherin immunostaining was done as above, and hormone immunostaining was then performed with specific guinea pig antibodies detected with Alexa

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488-conjugated donkey anti-guinea pig IgG (Molecular Probes). Sections were observed under a LSM510 Zeiss Confocal laser scanning microscope equipped with a krypton/argon mixed gas laser. The organization of the immunostained structures was studied on reconstructed images made by projecting 1-µm z-stacks of 2-3 consecutive confocal images. Unaltered digitalized images were transferred to a computer and Adobe Photoshop software was used to prepare final figures.

Binary Tree Analysis

Tumor analysis through binary trees (31) was performed using the *rpart* package in R (http://CRAN.R-project.org/package=rpart) (32). Invasiveness was used as output and 8 genes from 50 samples were used as predictors. Pruning of the tree was performed after leave-one-out cross-validation to prevent overfitting the tree to the training data. More information is available in the Appendix S1.

Statistical methods

Normal distribution of the variables was assessed by Shapiro-Wilk test. The comparisons between gene expression were done with Mann-Whitney U test. Reported correlations were performed using Spearman's correlation coefficient.

Results

Cadherin repertoire in human pituitary

Most studies on human pituitary adenomas have focused on E-cadherin expression and its role in tumorigenesis and tumor behavior. To investigate whether other members of the cadherin family could be involved in pituitary adenoma pathogenesis, we first examined the

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pattern of cadherin expression in normal human pituitary. We focused our study on type I and type II cadherins, characterized by a short ectodomain containing five extracellular cadherin repeats and two highly conserved intracellular domains required for beta-catenin and p120 catenin binding. Cadherin 13, a GPI-linked truncated isoform, and cadherin 16 and 17 containing 7 extracellular cadherin repeats (25) were also studied. We first quantified cadherin mRNA levels by real-time PCR. Figure 1A shows that among the 21 cadherins tested, *cadherin 5* (*CDH5*, specific from endothelial cells), *N-cadherin* (*CDH2*), *cadherin 18* (*CDH18*), and *E-cadherin* (*CDH1*) exhibit moderate to high levels of mRNA. Relative expression for *CDH5*, *N-cadherin*, *CDH18* and *E-cadherin* was 105 ± 35, 233 ± 66, 362 ± 79 and 696 ± 47, respectively. The other cadherins were either absent or expressed at very low levels. Since cadherins are associated with the regulatory intracellular components beta-catenin and p120 catenin, we also quantified their relative mRNA expression levels. Relative expression was 534 ± 73 for *beta-catenin* and 52 ± 10 for *p120 catenin* (data not shown). To establish the distribution of cadherin/catenin complexes within the pituitary gland, we performed immunohistochemistry on sections from normal pituitary double-stained for the different hormones secreted by the gland and either beta-catenin, p120 catenin, or the 3 major cadherins present in the gland, E-cadherin, N-cadherin and cadherin 18. Beta-catenin and p120 catenin were readily detected and broadly expressed throughout the gland. The staining was mainly present at the cell membrane and co-staining with the hormones showed that all endocrine cell types express these two catenins (data not shown). In contrast, cadherins displayed cell-type specific pattern of distribution (Fig 1B-P). Staining for E-cadherin and cadherin 18 was detected mainly at the plasma membrane from somatotrophs (Fig 1B and F) and lactotrophs (Fig 1C and G) and was either absent or present at low levels in corticotrophs (Fig 1H and N), thyrotrophs (Fig 1I and O) or gonadotrophs (Fig 1J and P). Conversely, N-cadherin staining was localized at the membrane in corticotrophs (Fig 1K), thyrotrophs (Fig

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1L) and gonadotrophs (Fig 1M) and barely detectable in somatotrophs (Fig 1D) and lactotrophs (Fig 1E). Thus, somatotrophs and lactotrophs express mainly E-cadherin and cadherin 18, whilst N-cadherin is most prominent in the other endocrine cell types.

Differential alteration of cadherin/catenin complexes in GH and PRL-secreting pituitary adenomas

We analyzed the cadherin expression profile in pituitary GH and PRL-secreting adenomas using q-PCR. We tested the 21 members and could not detect *de novo* expression of cadherins in tumors, only *E-cadherin*, *N-cadherin* and *cadherin 18* mRNAs were present. It appeared that *E-cadherin* mRNA levels were significantly reduced in invasive GH adenomas as compared to non-invasive tumors (Fig 2A). In 10 of 14 (71%) invasive GH adenomas, *E-cadherin* mRNA was barely detectable. *Cadherin18* mRNA levels were similar in non-invasive and invasive GH tumors (Fig 2B) and were not strikingly different from those quantified in normal pituitaries (Fig 1A). No significant differences were observed for *beta-catenin* and *p120 catenin* mRNA levels in non-invasive and invasive GH tumors (data not shown). We then examined the distribution of cadherin/catenin complexes in GH adenomas. In GH non-invasive tumors, the staining for E-cadherin, cadherin 18, beta-catenin and p120 catenin was mainly detected at the plasma membrane of the cells (Fig 2C-F). In contrast, the pattern of distribution of cadherin/catenin complexes was very different in GH invasive tumors, and was characterized by an absence of E-cadherin staining and low to strong cytoplasmic staining for cadherin 18, beta-catenin and p120 catenin (Fig 2G-J). Thus, the loss of E-cadherin is associated with a cytoplasmic redistribution of catenins, as previously reported in various tumor types (33), with a concomitant relocalization of cadherin 18 from the membrane to the cytoplasm.

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In contrast to GH adenomas, *E-cadherin* mRNA levels were not significantly different in non-invasive and invasive prolactinomas (Fig 3A). *Cadherin18* mRNA levels (Fig 3B), as well as *beta-catenin* and *p120 catenin* mRNA levels (data not shown), were comparable in non-invasive and invasive prolactinomas. In non-invasive prolactinomas, E-cadherin, cadherin 18, beta-catenin and p120 catenin immunostainings were mainly present at the plasma membrane of the cells (Fig 3C-F). The staining for cadherin/catenin complexes was different and heterogeneous in invasive prolactinomas (Fig 3G-N). Cadherin/catenin complexes were expressed at the plasma membrane in some part of the tissue section (Fig 3G-J). In some areas, E-cadherin membraneous staining was strongly reduced and the labeling was present in the cytoplasm (Fig 3K). The redistribution of E-cadherin was accompanied by a drastic alteration in the membraneous staining for cadherin 18, beta-catenin and p120 catenin (Fig 3L-N), and some cytoplasmic labeling was present for cadherin 18 and beta-catenin. Altogether, these results show that cadherin/catenin complexes were differentially regulated in GH and PRL adenomas.

Interestingly, *N-cadherin*, which is not detectable in GH and PRL cells in normal tissue, was expressed in a subset of GH and PRL adenomas. *N-cadherin* mRNA was present in 55% (15 of 27) of GH tumors (i.e. relative mRNA expression > 10; Fig 4A). Similarly, 72% (18 of 25) of prolactinomas expressed *N-cadherin* mRNA (Fig 4B). We performed immunohistochemistry to visualize N-cadherin distribution in these tumors expressing *N-cadherin* mRNA. N-cadherin staining was restricted to the cell membrane in non-invasive tumors (Fig 4C and E). In invasive tumors, although some cells exhibited a cytoplasmic N-cadherin staining, labeling was present predominantly at the plasma membrane (Fig 4D and F). In addition, it is worth noting that N-cadherin positive cells exhibited morphological changes in invasive tumors, reminiscent of the profound structural modifications affecting cells which undergo EMT (34). Indeed, in invasive tumors, N-cadherin positive cells

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displayed an irregular, elongated mesenchymal shape, while they showed an epithelial phenotype in non-invasive tumors.

Discrimination of GH and PRL-secreting adenomas based on invasive behavior by combining cadherin repertoire with markers of invasion

In an attempt to discriminate tumors on the basis of invasiveness, and to evaluate the importance of the expression profiles of cadherin/catenin complexes in determining tumor phenotype, we combined the patterns of expression of these adhesion molecules with those of a subset of well-established pituitary adenoma markers. We first examined the expression profiles of *ESRP1*, *LGALS3* and *PTTG1*. ESRP1 (Epithelial Splicing Regulatory Protein 1) has been recently proposed to act as a key regulator of the EMT process in GH adenomas (22). Galectin 3 (LGALS3) is expressed in PRL and ACTH adenomas (35), and two recent studies have proposed that it is a strong predictive factor of recurrence/progression of PRL adenomas (36, 37). PTTG1 (Pituitary Tumor Transforming Gene 1) plays an important role in pituitary tumorigenesis, is overexpressed in pituitary adenomas and has been proposed to be a biomarker of aggressive pituitary adenomas (15, 38).

Fig 5A shows that in GH-secreting adenomas, *ESRP1* expression was significantly reduced in invasive tumors compared to non-invasive adenomas. *ESRP1* was also present in prolactinomas and its expression was related to tumor invasiveness (Fig 5D). In a recent study of GH adenomas, it was reported that *ESRP1* and *E-cadherin* mRNAs were correlated and that silencing of *Esrp1* in GH3 cells induced a significant decrease in *E-cadherin* expression (22). We found that in GH-secreting adenomas *E-cadherin* expression was positively associated with *ESRP1* expression ($R = 0.87$, $P < 0.0001$), while no correlation was observed in prolactinomas ($R = 0.33$, $P = 0.112$). In addition, we observed no significant

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association between *LGALS3* and *PTTG1* expression and tumor invasiveness in both types of adenomas (Fig 5B, C, E and F).

We then analyzed the gene expression profiles to establish binary trees of adenomas based on the invasiveness behavior of the tumors. The binary tree for tumor invasion is shown on Fig 6A and indicates that different patterns of gene expression allow distinct separation of invasive and non-invasive tumors with an acceptable misclassification rate (12%). The results indicate that the cadherin repertoire plays a significant role in distinguishing GH and PRL tumors in regards to their invasive behavior. The importance of each gene for the construction of the binary tree is illustrated in Fig 6B and shows that *ESRP1* and *E-cadherin* are the major genes distinguishing the invasiveness behavior of the tumors.

Discussion

We report here that human normal pituitary gland exhibits a limited and cell-type specific cadherin expression profile. In tumors, this cadherin repertoire undergoes major remodeling and is differentially altered in GH-secreting adenomas and prolactinomas. E-cadherin expression is significantly reduced in invasive GH tumors and is correlated with a cytoplasmic redistribution of cadherin/catenin complexes. In invasive prolactinomas, E-cadherin distribution is reshaped and is associated with a concomitant disorganization of cadherin/catenin complexes. In addition, a subset of adenomas has altered expression of N-cadherin, and morphological changes evocative of EMT-like process are peculiar to invasive tumors. Using binary tree analysis, we demonstrate that this class of adhesion molecules, in association with known pituitary adenoma markers, allows discrimination of tumors by their invasive behavior.

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The investigation of the cellular expression and distribution of more than 20 members of the superfamily of cadherin proteins has shown that the cadherin repertoire of normal human pituitary is restricted, since only E-cadherin, N-cadherin and cadherin 18 were detected. This is in agreement with previous studies of the expression of E- and N-cadherin in normal tissue (39-42), although in these, the cellular expression of E- and N-cadherin within the normal gland was either not investigated or incomplete for the different endocrine cell types. Here we have included evaluation of the cellular distribution of these adhesion molecules and showed that the cadherin expression pattern is cell-type specific. Somatotrophs and lactotrophs express mainly E-cadherin and cadherin 18, while N-cadherin is present in the other endocrine cell types. Therefore, even if the cadherin repertoire is quite different in humans and rodents it appears that the different cellular types of the human pituitary gland can be characterized by a defined combinatorial expression of different subsets of cadherins as previously reported in the murine pituitary (27).

This repertoire of adhesion molecules is highly altered in tumors and, interestingly, GH-secreting adenomas and prolactinomas display differential modulation of cadherin/catenin complex expression. Such patterns of expression specific of tumor type have been reported for somatostatin receptor subtypes (7). These results suggest that, although GH and PRL cells derive from the same lineage (43), tumorigenesis process involves a multiplicity of underlying mechanisms probably specific for the tumor subtype. It is worth noting that these two types of tumors do not present *de novo* expression of the cadherins not detected in the normal gland. We show that *E-cadherin* mRNA levels were not significantly different in invasive and non-invasive prolactinomas. In contrast, *E-cadherin* mRNA levels were significantly reduced in invasive compared with non-invasive GH-secreting tumors. Alteration of E-cadherin expression has been shown in pituitary adenomas (8, 19, 40), ~~and~~ is now well characterized in GH-producing adenomas (8, 42, 44, 45) and is not correlated to

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pre-operative medical treatment (8). In our study, we have observed that *ESRP1* expression was significantly reduced in GH invasive tumors and *E-cadherin* expression was positively associated with that of *ESRP1*. This is in good agreement with a previous study reporting that *ESRP1*, an important regulator of EMT, correlates with *E-cadherin* mRNA in a large cohort of patients with acromegaly (22). We have extended this analysis since we have shown that *ESRP1* is also expressed in prolactinomas with similar levels than in GH-secreting tumors, however no correlation between *ESRP1* and *E-cadherin* was observed in prolactinomas, suggesting that distinct regulatory mechanisms for *E-cadherin* expression might take place in both types of tumors.

In approximately 70% of PRL and 55% of GH adenomas we observed expression of *N-cadherin*, a cadherin expressed in the normal gland by endocrine cell types other than GH and PRL cells. Interestingly, *N-cadherin* expression was previously reported by Rubinek *et al.* (46) in 8 of 12 GH adenomas and 1 of 7 prolactinomas. The inappropriate up-regulation of N-cadherin in some cancer cells has been shown to promote motility and invasion (47, 48), and induces major effects on cell phenotype and behavior (16, 18). A high level of N-cadherin expression is often associated with poor prognosis (16). In our study, N-cadherin positive cells adopt morphological features consistent with a mesenchymal phenotype in invasive tumors, suggesting that an EMT-like process might occur in some pituitary adenomas and might be associated with GH and PRL progression. Epithelial cells that undergo EMT are transcriptionally reprogrammed, which in turn leads to decreased adhesion, enhanced migration or invasion and increased resistance to apoptosis (49). Previous cellular and molecular studies suggest that potential EMT might take place in pituitary tumors (21, 22, 50), the morphological changes that we report here in invasive tumors strengthen these results.

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The differential alterations of cadherin/catenin complex expression in invasive and non-invasive adenomas led us to analyze this class of adhesion molecule as a relevant parameter for the discrimination of pituitary tumors based on the invasive behavior. To address this point, we have chosen a gene candidate approach by analyzing cadherin expression in association with the pattern of expression of known molecular markers related to pituitary tumor behavior and susceptibility to organize tumors. Of note, the number of genes used in binary tree analysis is not limited and emerging studies of exome array analysis in pituitary adenomas (51, 52) would enable to increment the number of genes in future studies. Such strategies could be applied to invasive behavior, resistance to dopamine agonists or somatostatin receptor analogues, tumor recurrence... The binary tree based on the invasive behavior of the tumors shows that the two types of tumors can be distinguished regarding their pattern of gene expression with a low misclassification rate. *ESRP1* and *E-cadherin* play an important role in the establishment of the binary tree. Thus, these results show that combining the pattern of cadherin expression with known biomarkers can discriminate the invasive behavior of pituitary adenomas. Cadherin/catenin complexes are significant factors to separate invasive from non-invasive GH and PRL tumors.

We report here that cadherin repertoire contributes to the discrimination of GH and PRL adenomas in regards to tumor invasiveness. Since we have shown that cadherin profiles are cell-type specific, it would be of interest to examine whether this class of adhesion molecules could be useful to analyze other pituitary adenoma subtypes. In addition, as prediction of pituitary tumor recurrence or invasiveness is problematic, future studies determining if tumors exhibiting *de novo* expression of N-cadherin have a tendency to recur could bring new insights in the research of prognosis biomarkers. Finally, it would be interesting to elucidate the role of N-cadherin in pituitary adenoma pathogenesis, especially

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in tumors with induced expression of N-cadherin, since it is possible that the invasion process in this type of tumors relies on N-cadherin up-regulation.

In conclusion, we show here that the cadherin expression profile in normal human pituitary gland is restricted and cell-type specific, and displays major differential alterations in GH and PRL tumors. A subset of GH and PRL tumors express N-cadherin, and morphological changes are suggestive of EMT-like process and are unique to invasive tumors. The association with known molecular markers shows that this cadherin repertoire plays a substantial role in the discrimination of GH and PRL tumors based on tumor invasive behavior.

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Figure legends

Fig 1. Cadherin expression profile and cellular distribution in normal human pituitary gland. (A) Cadherin mRNA levels were quantified by real time PCR and results are presented as mean ± SEM. *E-cadherin (CDH1)*, *N-cadherin (CDH2)* and *cadherin 18 (CDH18)* exhibited the highest levels of expression. (B-P) CDH1, CDH2 and CDH18 (red) distribution in hormone-producing cells (green) of normal human pituitary gland. (B, D and F) Confocal images showing double immunofluorescence labeling of pituitary sections with specific antibodies against GH and CDH1 (B), CDH2 (D) or CDH18 (F). (C, E and G) Confocal images showing double immunofluorescence labeling of pituitary sections with specific antibodies against PRL and CDH1 (C), CDH2 (E) or CDH18 (G). To improve cadherin staining visibility, the red channel is shown alone on the left. (H-P) Confocal images showing double immunofluorescence labeling of pituitary sections with specific antibodies against ACTH (H, K and N), TSH (I, L and O) or LH-FSH (J, M and P) and CDH1 (H-J), CDH2 (K-M) or CDH18 (N-P). CDH1 and CDH18 were mainly detected in somatotrophs and lactotrophs, whilst CDH2 was prominent in corticotrophs, thyrotrophs and gonadotrophs. Scale bar, 20 µm.

Fig 2. *E-cadherin* and *cadherin 18* mRNA expression and cadherin/catenin complex distribution in GH-secreting adenomas. (A and B) Cadherin mRNA levels were quantified by real time PCR in non-invasive (NI, n = 13) and invasive (I, n = 14) GH-secreting adenomas. Results are presented as box plots with median value (line), the interquartile range (box) and the range of the data. *** $P < 0.001$. (C-J) Confocal images showing immunofluorescence labeling of non-invasive (C-F) and invasive GH-secreting adenomas (G-J) with specific antibodies against E-cadherin (C and G), cadherin 18 (D and H), beta-catenin (E and I) and p120 catenin (F and J). Scale bar, 20 µm.

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Fig 3. *E-cadherin* and *cadherin 18* mRNA expression and cadherin/catenin complex distribution in PRL-secreting adenomas. (A and B) Cadherin mRNA levels were quantified by real time PCR in non-invasive (NI, n = 17) and invasive (I, n = 8) prolactinomas. Results are presented as box plots with median value (line), the interquartile range (box) and the range of the data. (C-N) Confocal images showing immunofluorescence labeling of non-invasive (C-F) and invasive prolactinomas (G-N) with specific antibodies against E-cadherin (C, G and K), cadherin 18 (D, H and L), beta-catenin (E, I and M) and p120 catenin (F, J and N). Scale bar, 20 μm.

Fig 4. *N-cadherin* mRNA expression and distribution in GH-secreting adenomas and prolactinomas. (A and B) *N-cadherin* mRNA levels were quantified by real time PCR in non-invasive (NI, n = 13) and invasive (I, n = 14) GH-secreting adenomas (A), and in non-invasive (NI, n = 17) and invasive (I, n = 8) prolactinomas (B). Results are presented as box plots with median value (line), the interquartile range (box) and the range of the data. (C-F) Confocal images showing immunofluorescence labeling of non-invasive and invasive GH-secreting adenomas and prolactinomas with N-cadherin antibody. Note the epithelial morphology of N-cadherin positive cells in non-invasive tumors (C and E) and the elongated and irregular shape of tumoral cells in invasive tumors (D and F). Scale bar, 20 μm.

Fig 5. *ESRP1*, *LGALS3,* and *PTTG1* mRNA expression in GH-secreting adenomas and prolactinomas. (A-C) Genes of interest were quantified by real time PCR in non-invasive (NI, n = 13) and invasive (I, n = 12) GH-secreting adenomas. (D-F) Genes of interest were quantified by real time PCR in non-invasive (NI, n = 17) and invasive (I, n = 8) prolactinomas. Results are presented as box plots with median value (line), the interquartile range (box) and the range of the data. * $P < 0.05$, ** $P < 0.01$.

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Fig 6. Binary trees of GH-secreting adenomas and prolactinomas based on the invasiveness behavior of the tumors. (A) Classification tree for tumor invasiveness. Numbers underneath the leaves show the number of invasive and non-invasive tumors in each group (I/NI). The misclassification rate of this tree is 12%. (B) Importance of each gene for the classification, as a measure of enhancement of branches associated with that gene.

Supporting Information

The following supplementary material is available:

Table S1. Patient clinical characteristics.

Table S2. Human primer sequences used for real-time PCR.

Appendix S1. Protocol for binary tree analysis.

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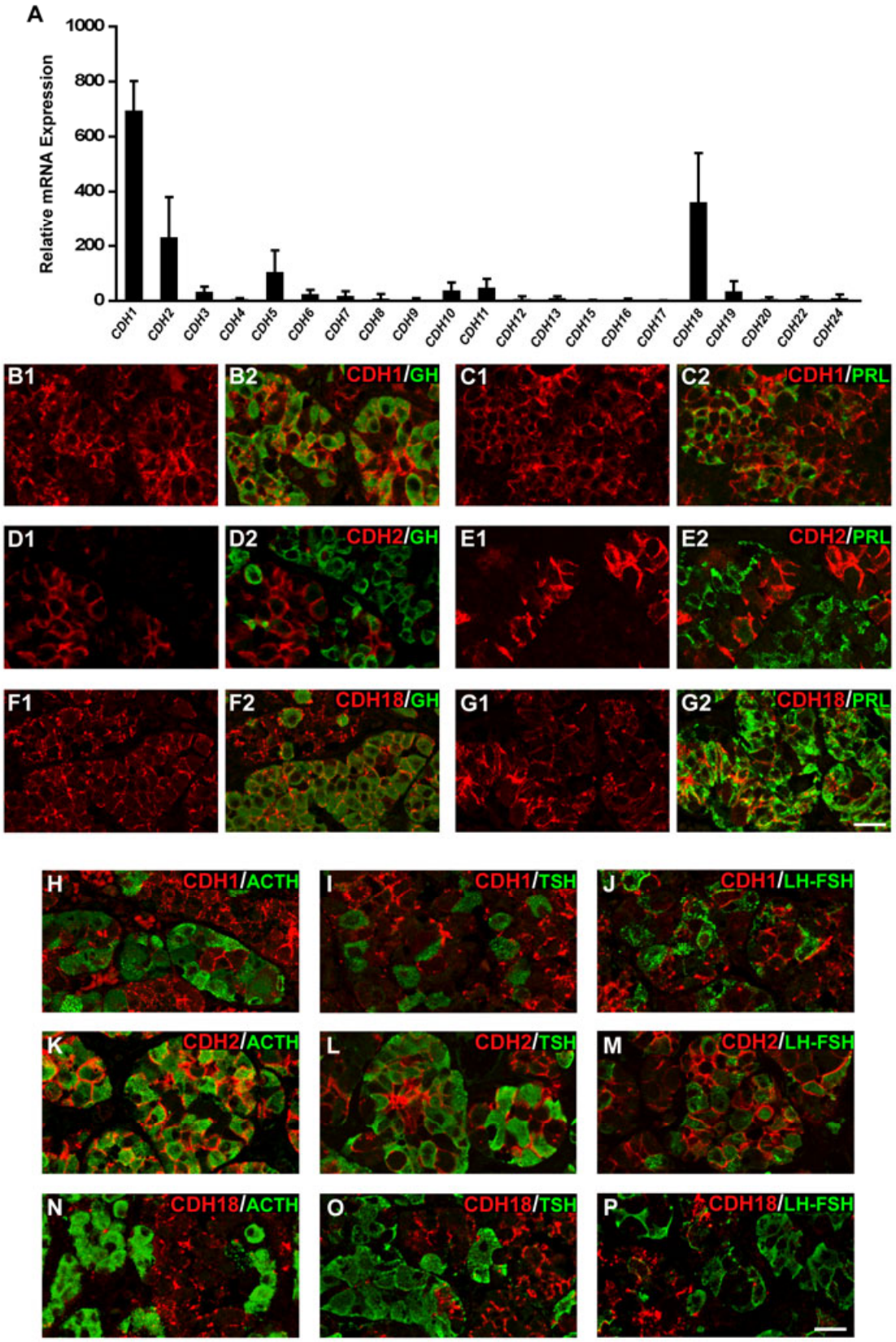

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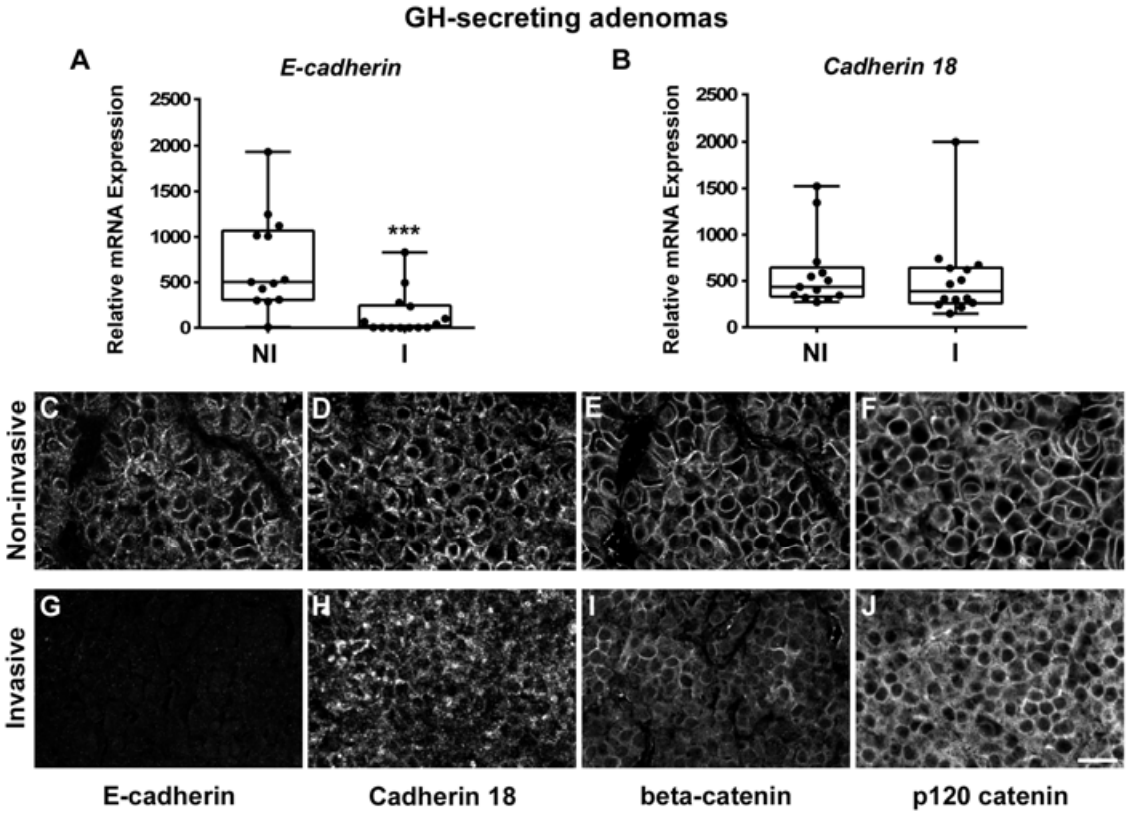

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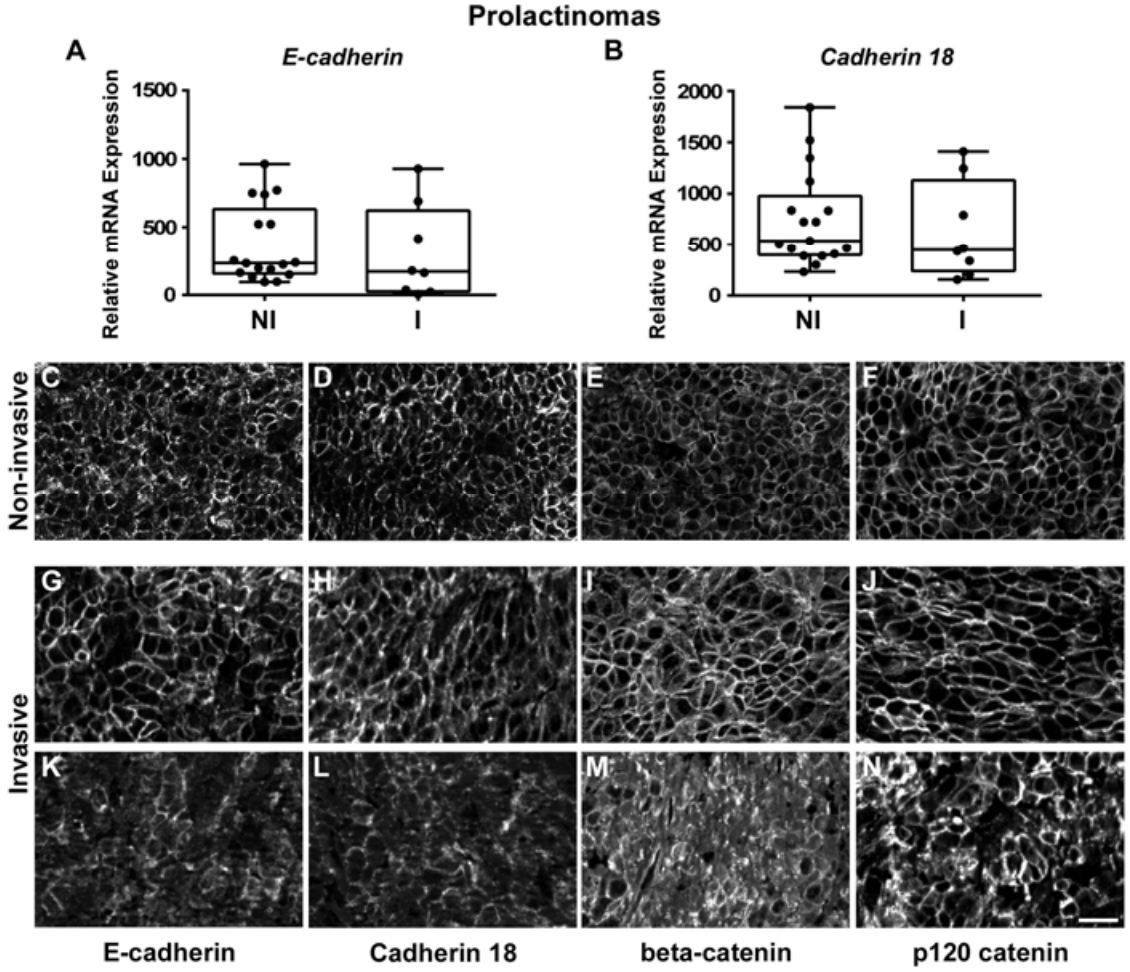

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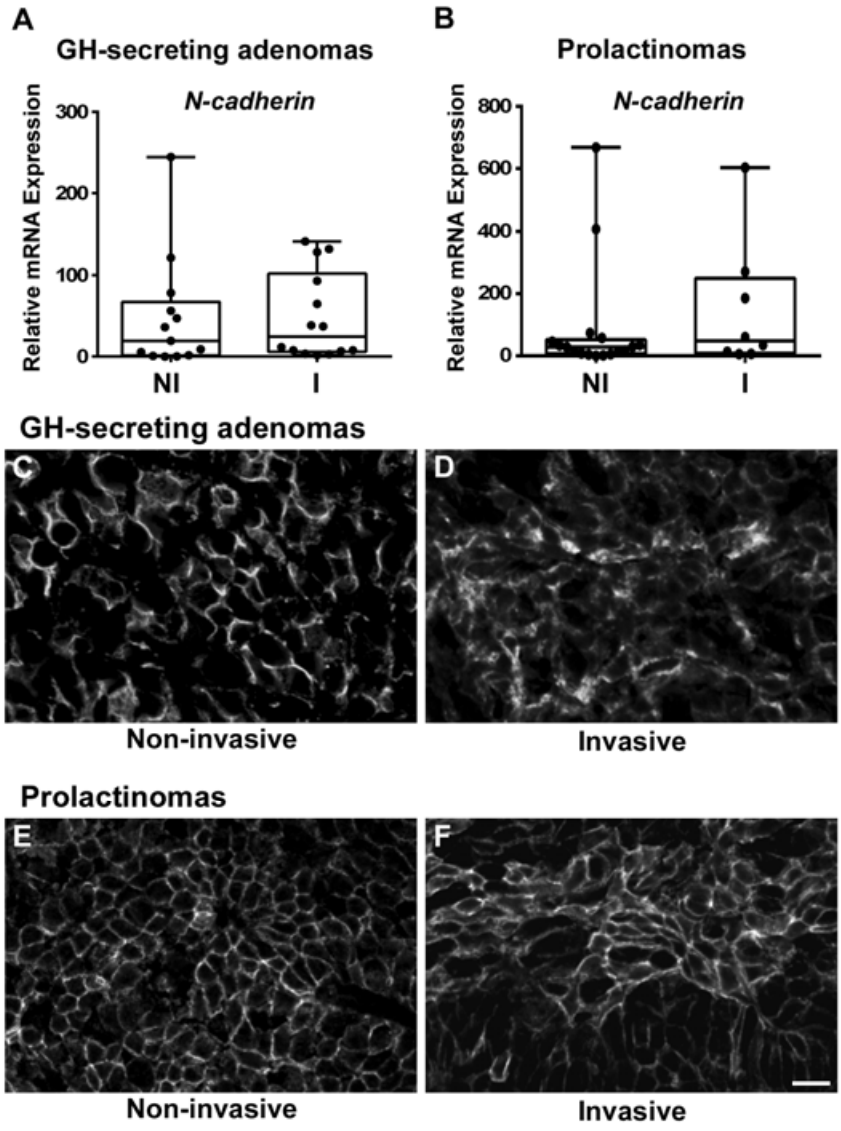

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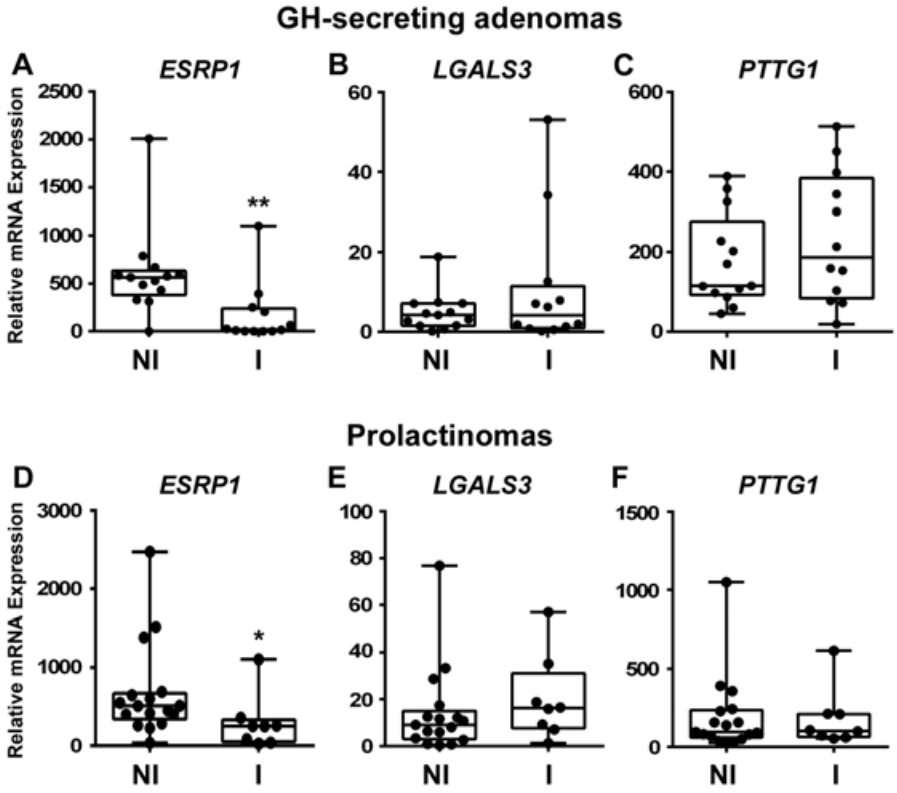

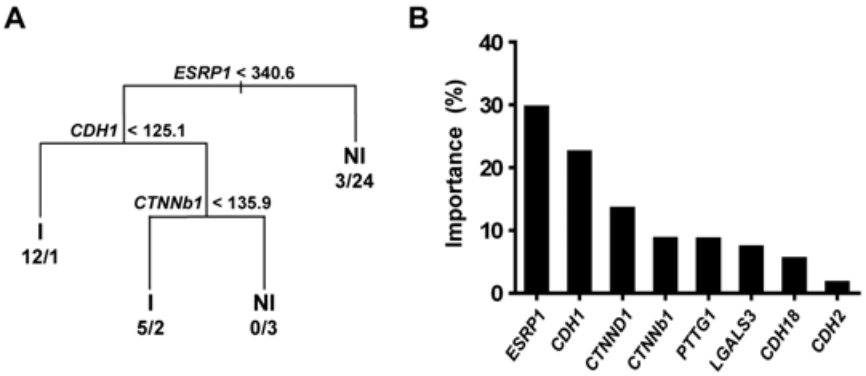

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